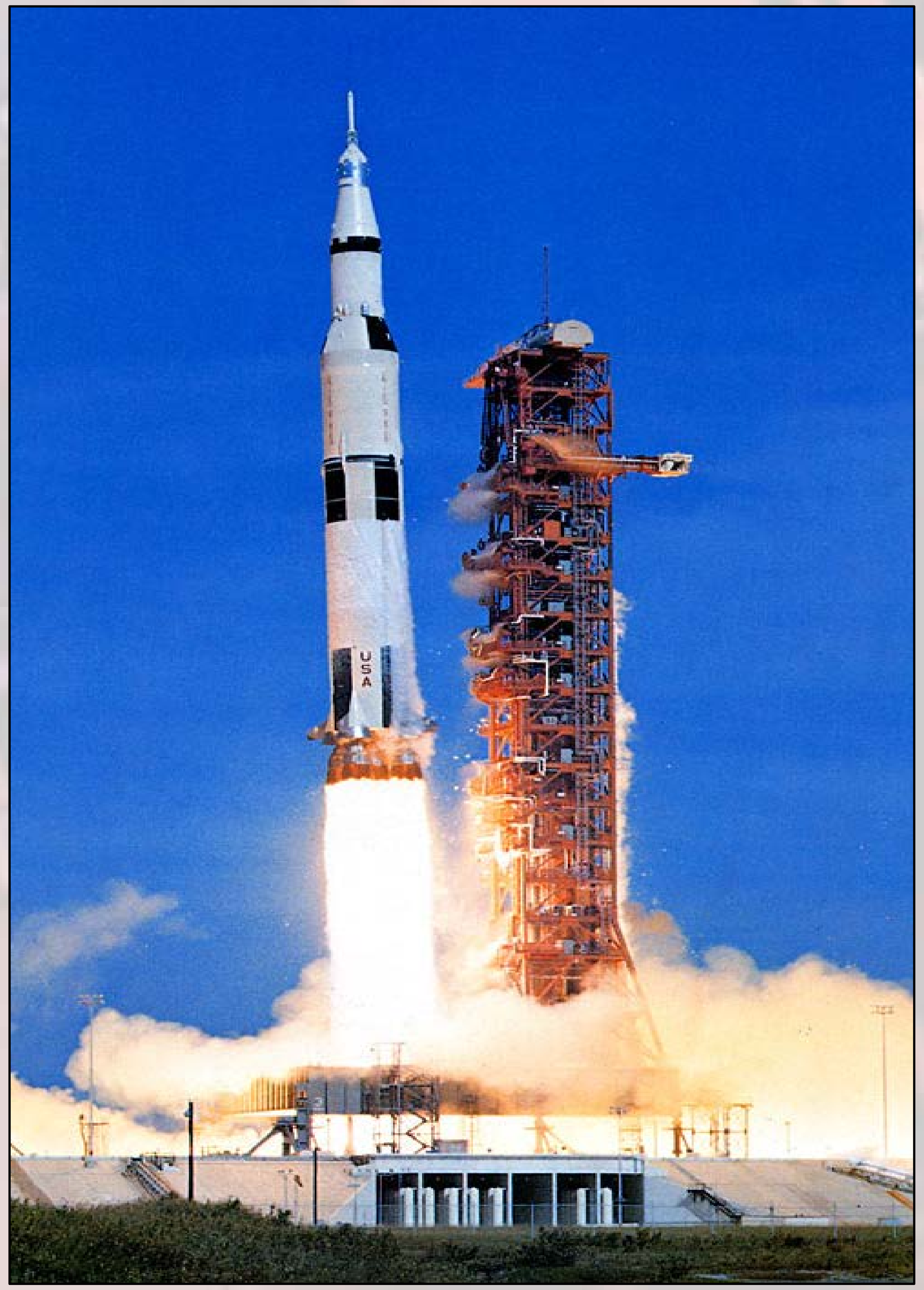
Development of Storage Methods for *Saccharomyces* Strains to be Utilized for *In situ* Nutrient Production in Long-Duration Space Missions

Natalie Ball, KBR Wyle
Hiromi Kagawa, SETI Institute
Aditya Hindupur, KBR Wyle
John Hogan, NASA Ames Research Center

From Sea to Space

Nutrient deficiencies occur as a result of limited resupply of fresh foods during long-duration expeditions

STS-110 crew eating on board the International Space Station



Nutrient Degradation Over Time

Nutritional quality of 109 space food items tested over three years at ambient temperature storage

Nutrients below the recommended intake post-processing	Calcium	Potassium	Vitamin K	Vitamin D
Vitamins that may degrade to lower than the recommended daily intake after three years	Vitamin B1	Vitamin C	Vitamin B9*	

* Vitamin degradation dependent on food source

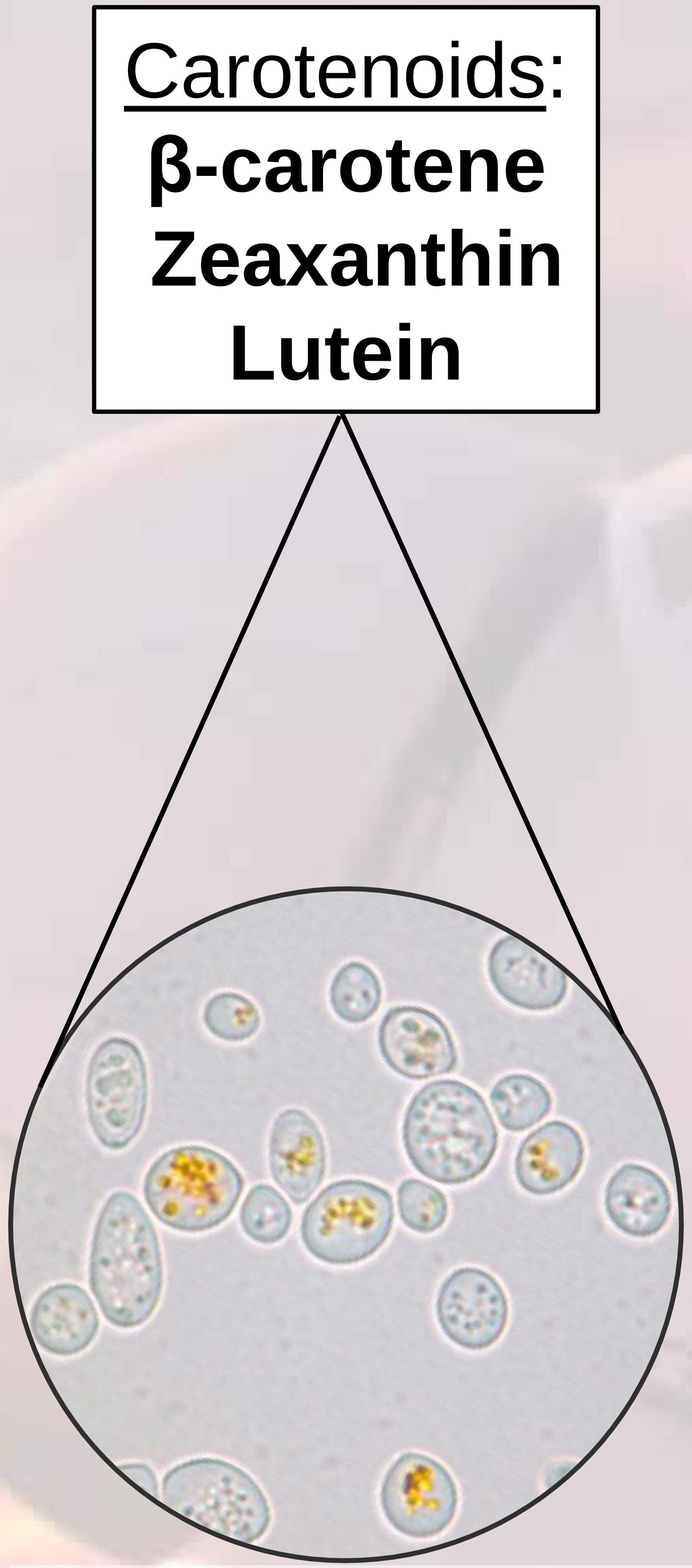
Cooper, Maya, Michele Perchonok, and Grace L. Douglas. "Initial assessment of the nutritional quality of the space food system over three years of ambient storage." *npj Microgravity* 3.1 (2017): 17.

Microorganisms for *In situ* Production of Nutrients

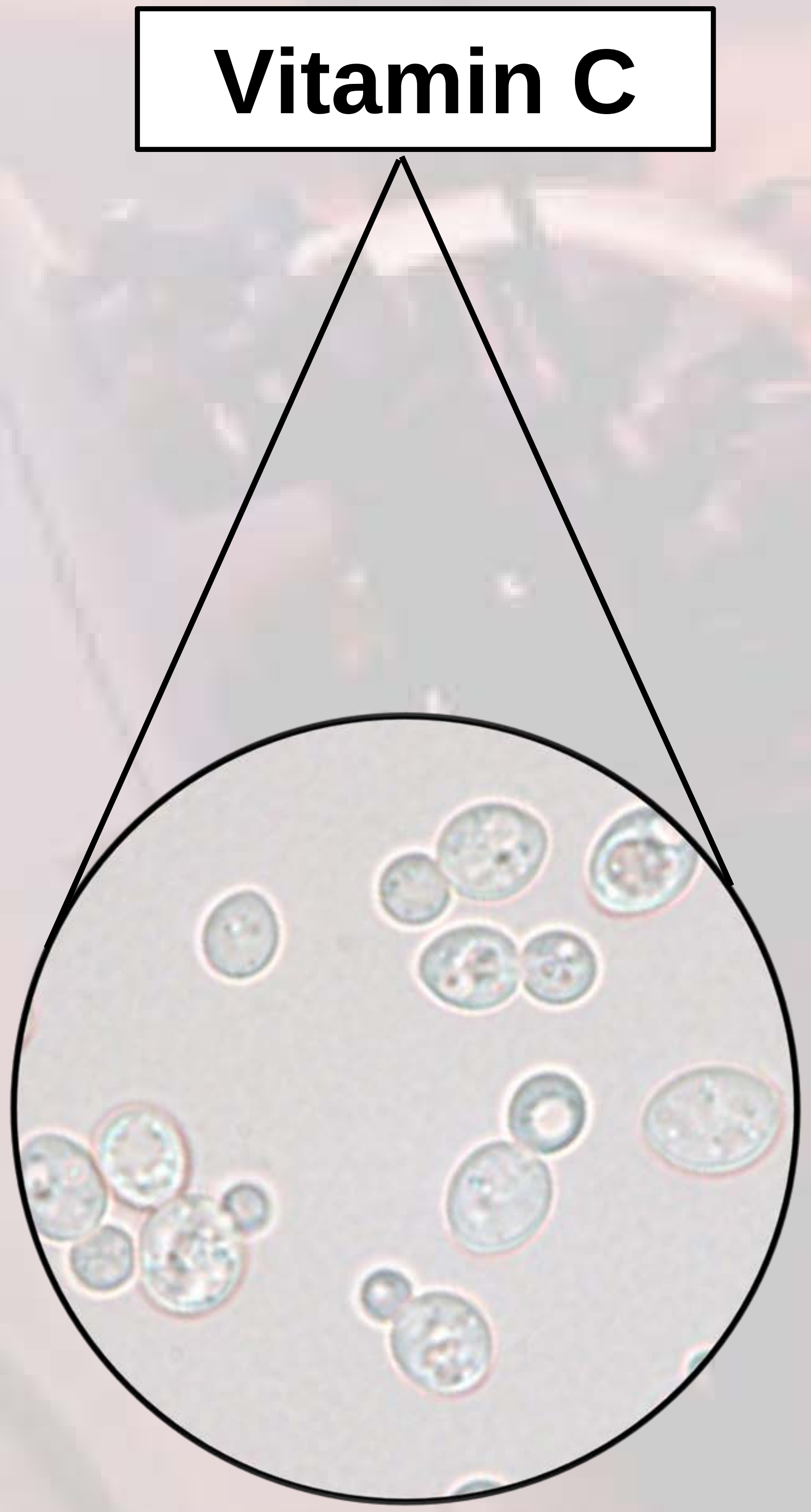
In order for *In situ* production of nutrients to occur microorganisms must maintain high viability during long-duration storage

Nutrient	Recommended Dietary Intake (RDI)[2]	Published Nutrient Yields
Vitamin C	75 – 90 mg/day	~100 mg/L[3]
Vitamin K	90 –120 µg/day	85 µg/g wet weight[4]
Beta-carotene (provitamin A)	6 – 16 mg/day	5.9 mg/g dry cell weight[5]

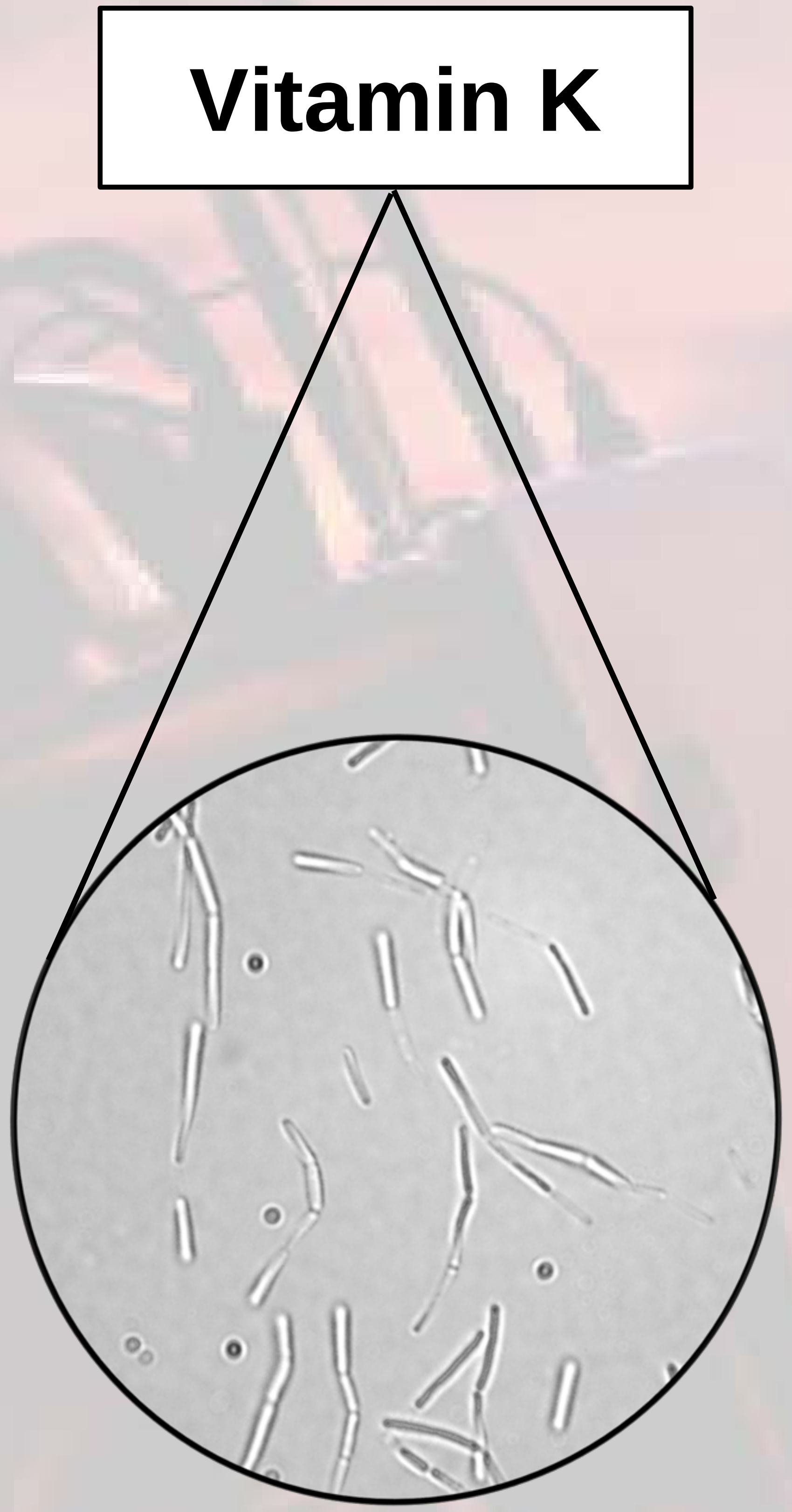
Citations: [2]*Code of Federal Regulations, title 21, Sec 101.9,*
[3]Sauer et al., 2004, [4]Yanagisawa and Sumi, 2005, [5]Verwaal et al., 2007



Saccharomyces cerevisiae boulardii (expressing β-carotene)



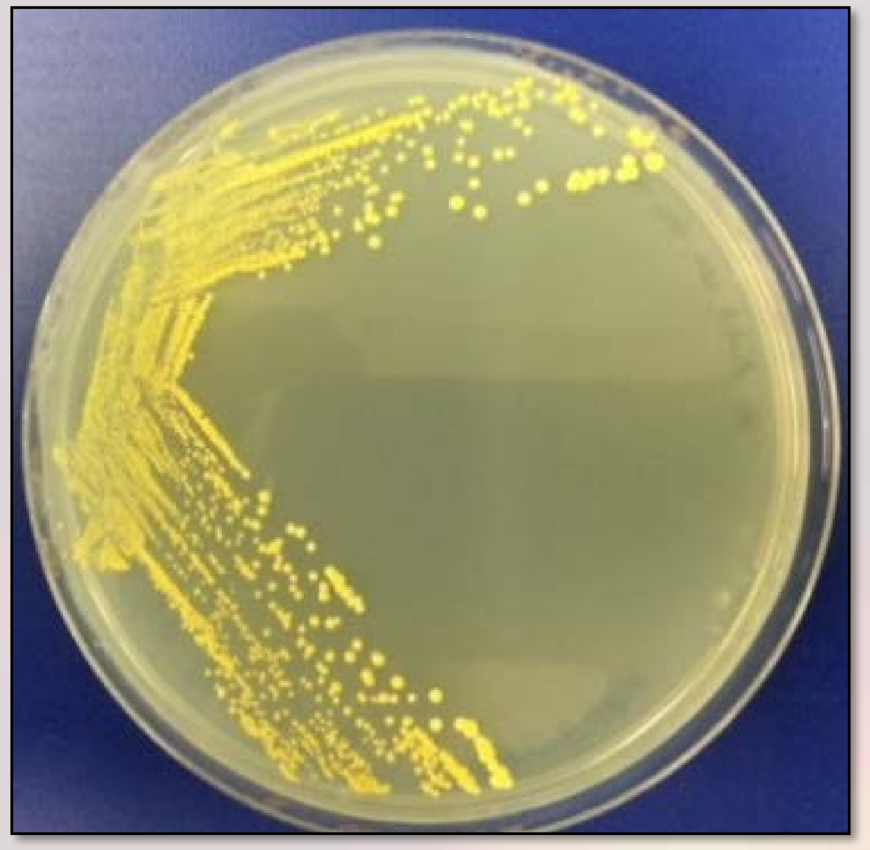
Saccharomyces cerevisiae



Bacillus subtilus

BioNutrients Project

Objective: To engineer a GRAS (generally regarded as safe) microorganism for the *In situ* production of needed dietary nutrients for long-duration space missions



Carotenoid producing strain

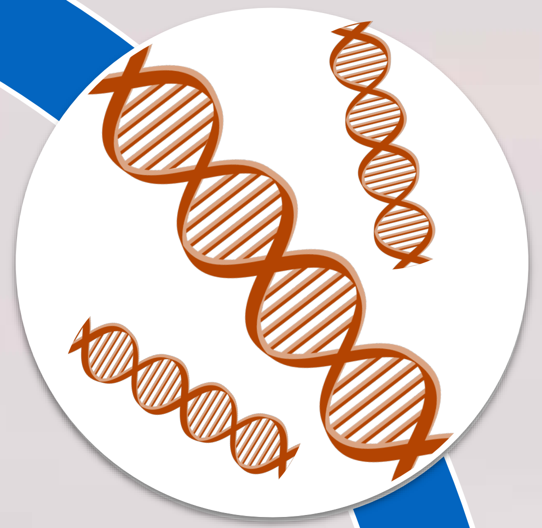
Desiccation (Air-dry)

Long-term storage

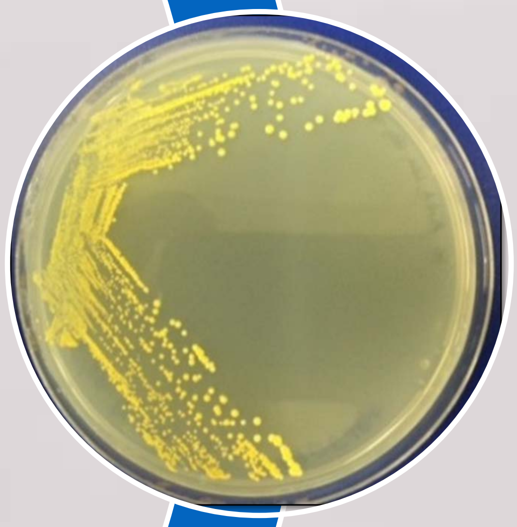
Revival in edible media

Yeast as *In-situ* Production Platform

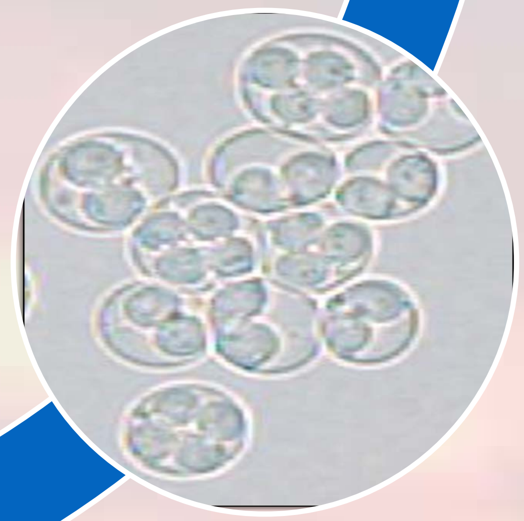
S. cerevisiae



History of Metabolic Engineering



Expression Platform Organism

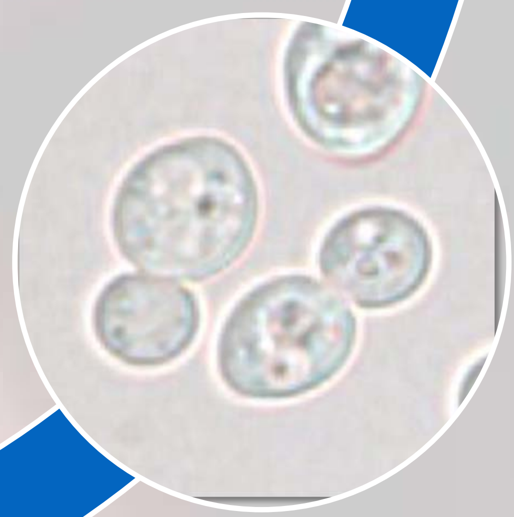


Spore Former

S. boulardii

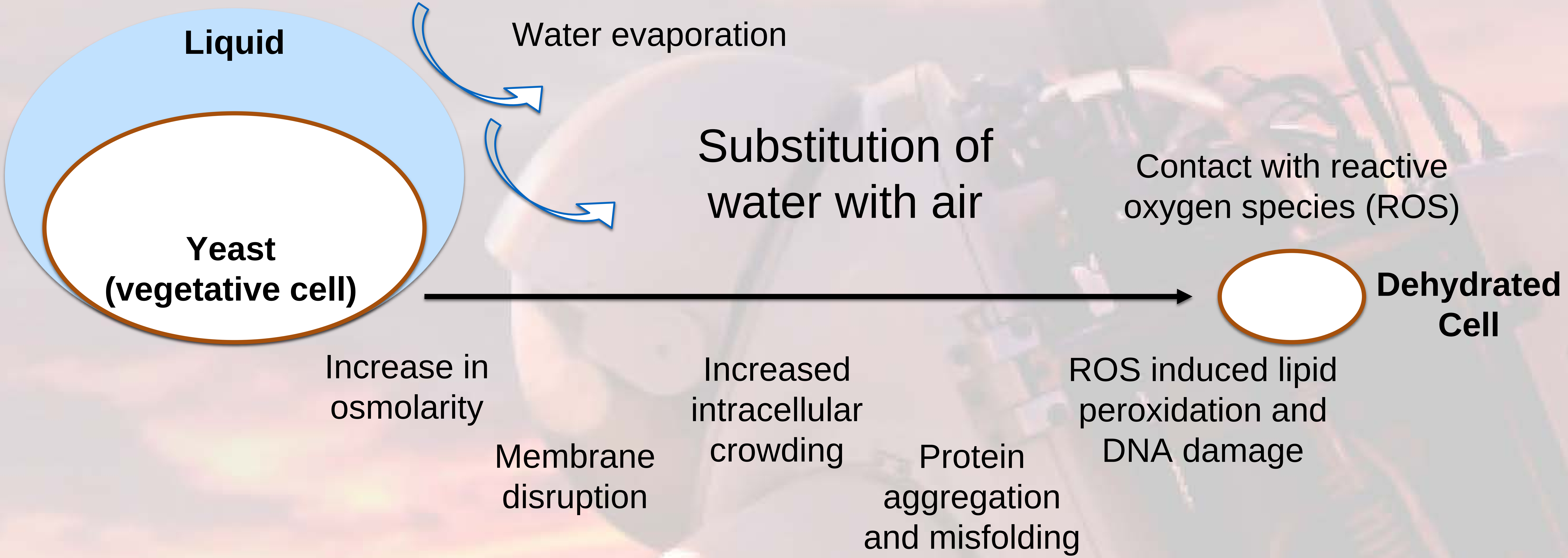
Same Engineering Tools can be Applied

Probiotic



Vegetative Cells

Effects of Dehydration on Yeast



Dupont, Sebastien, et al. "Survival kit of Saccharomyces cerevisiae for anhydrobiosis." *Applied microbiology and biotechnology* 98.21 (2014): 8821-8834.

Preservation of Spores and Vegetative Cells

Lyophilizer

Vacuum

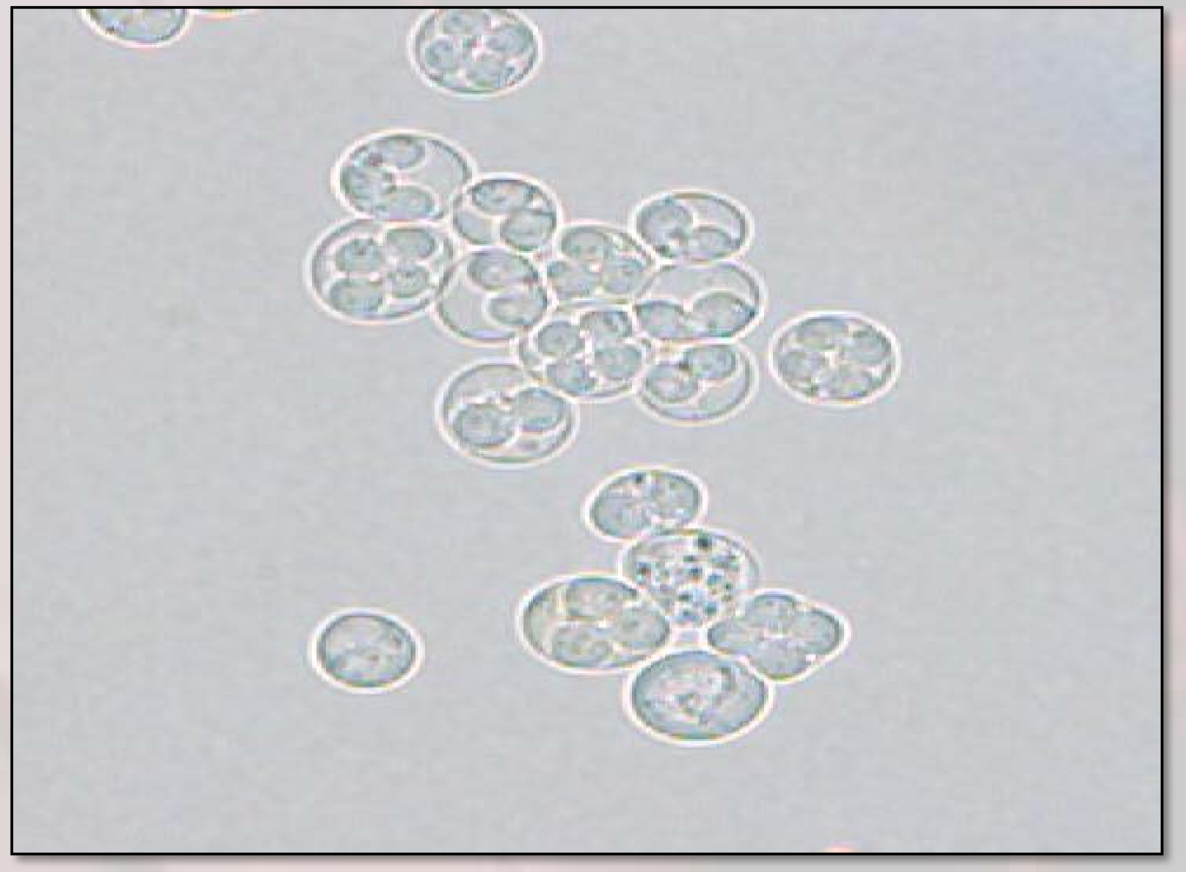
Drying Methods

- Lyophilization (freeze-dry)
- Vacuum (no freezing involved)
- Air-dry

Protectants

- The following protectants are identified as edible and have proven successful:

  – Trehalose, skim milk, monosodium glutamate
  – Proline
  – Sorbitan monostearate
  – Lactose



Storage

- Stored in reduced oxygen environment at room temperature or 4 ^{0}C

Methods Flowchart

Vegetative Cells:
Saccharomyces cerevisiae and *boulardii*

Desiccation
- Lyophilization
- Air-drying

Storage
- Samples stored in an anaerobic chamber in 96 well plates at room temperature

Revival
- Rehydrated in dilute PBS for 30 minutes, serially diluted, plated, and CFU counted

Spores:
Saccharomyces cerevisiae

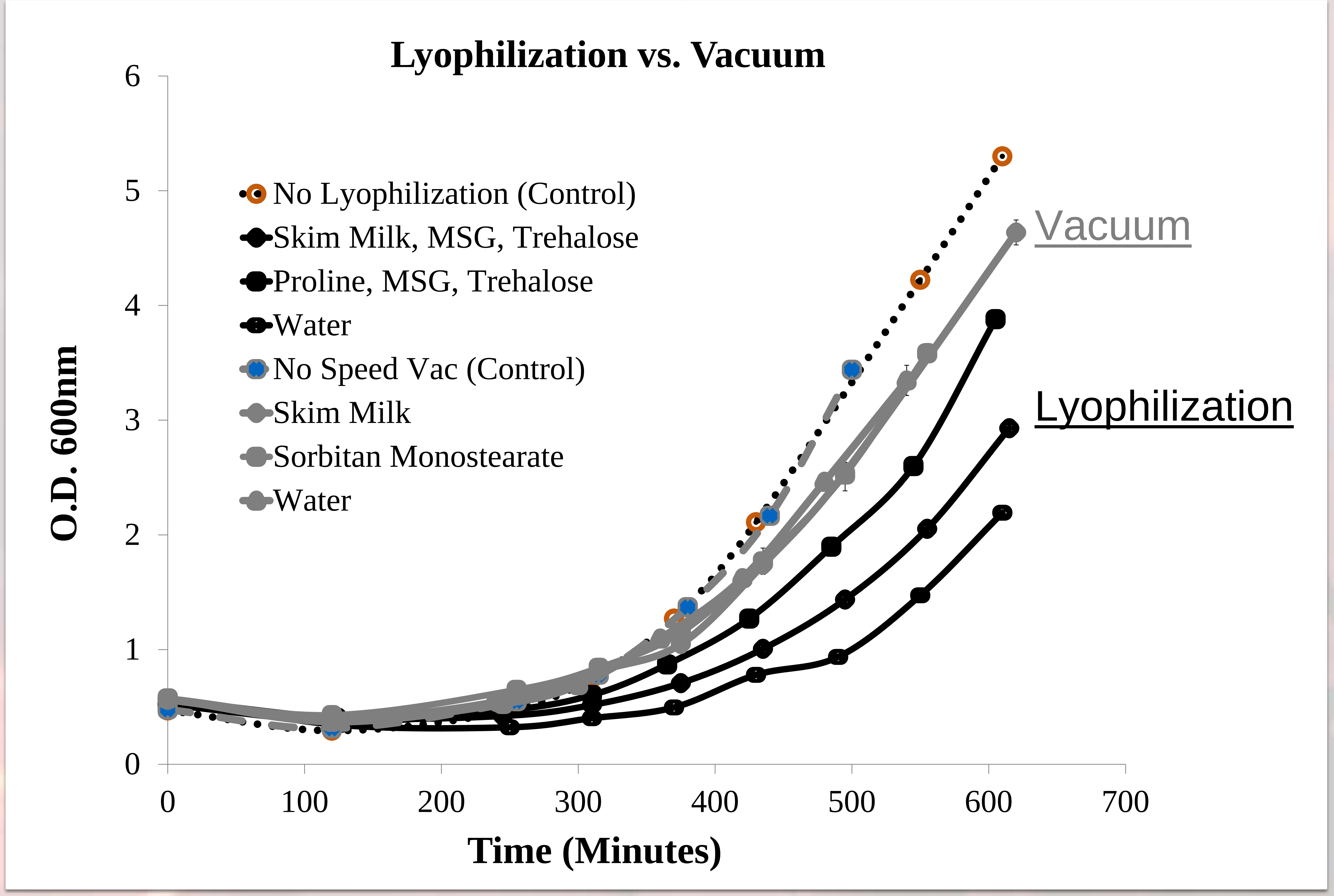
Desiccation
- Lyophilization
- Vacuum
- Air-dry

Storage
- Sealed in bags without oxygen, and stored at room temperature or 4 ^{0}C

Revival
- Measured by optical density
- Measured by percent change in biomass

Effect of Drying Methods on Spore Survival

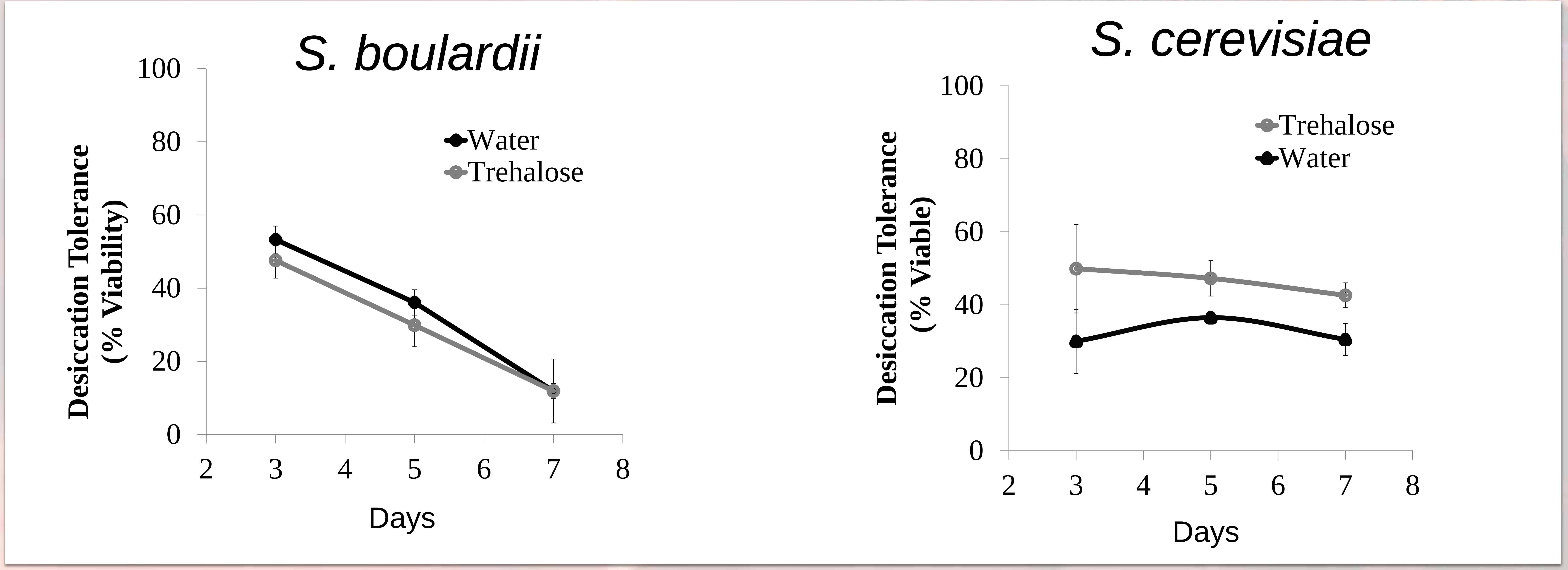
- Protectants did not affect spore survival under vacuum at room temperature
- Protectants increased viability of lyophilized spores
- Lyophilization was overly damaging to spores when compared to vacuum



Optimizing Vegetative Cell Viability

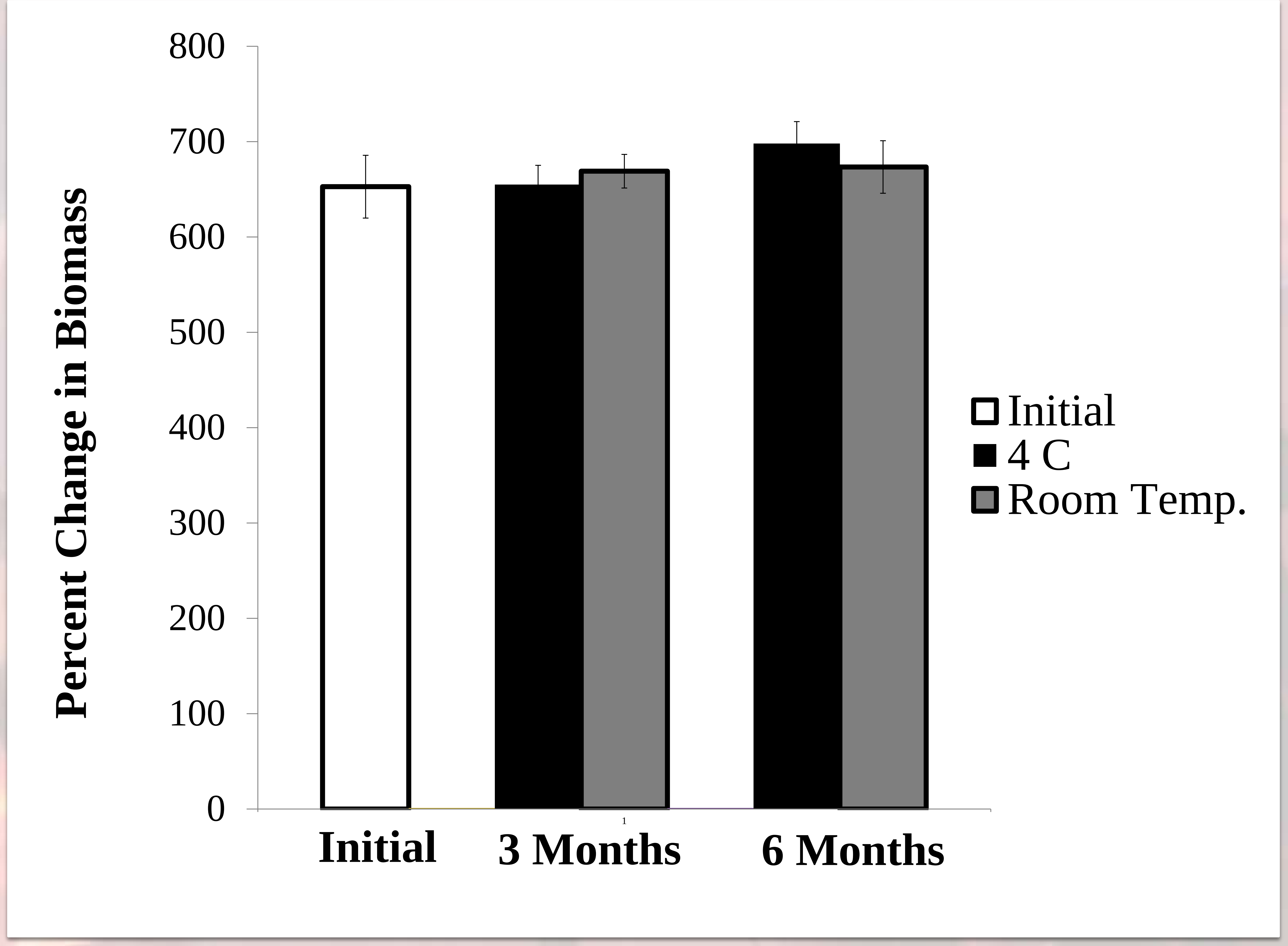
Vegetative cells were allowed to grow in rich media for 3, 5, and 7 days to determine if time spent in stationary phase had an effect on viability after desiccation

<u>Tested with trehalose as a protectant</u>



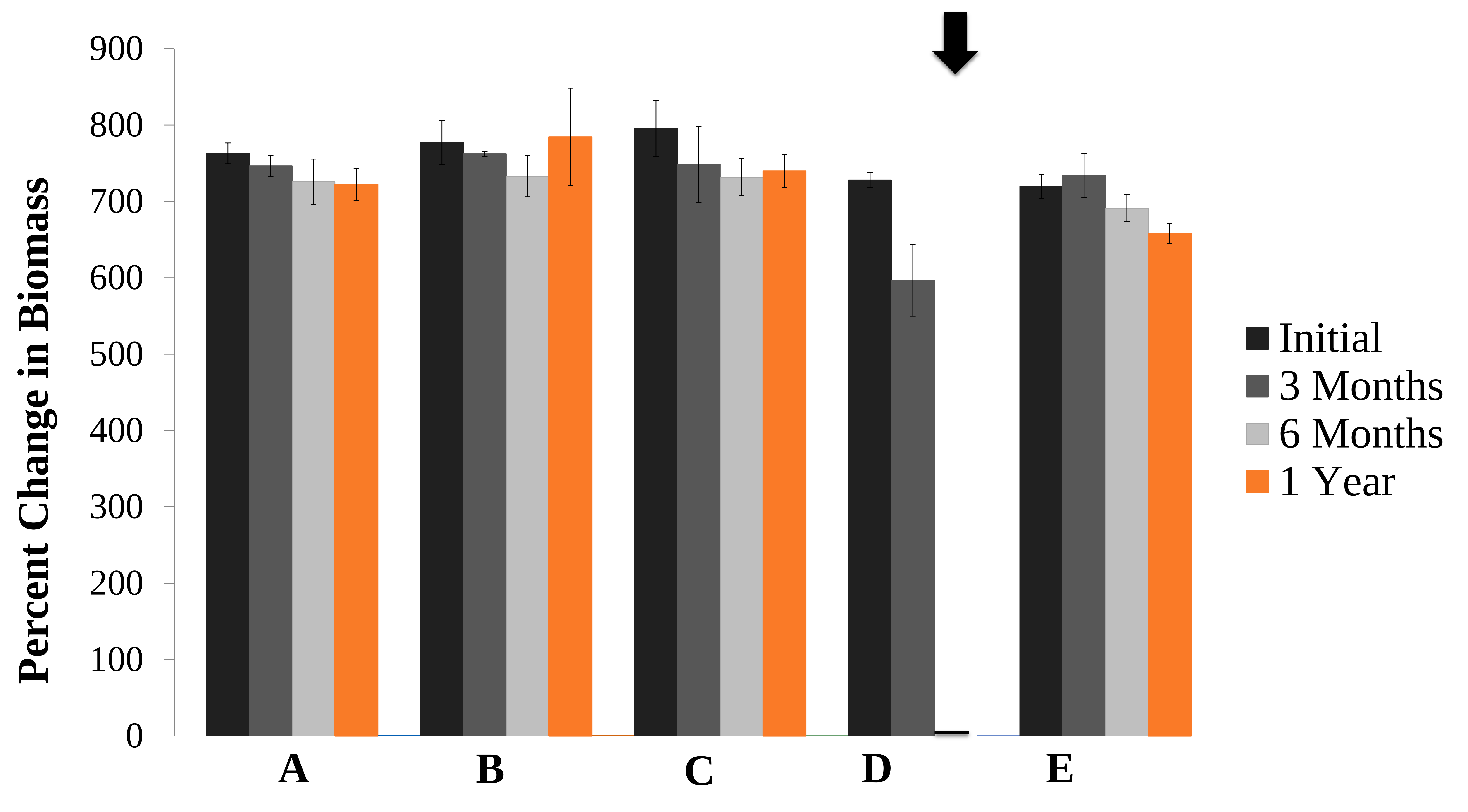
Viability of Spores Stored at 4 ºC

- Spores stored at room temperature or at 4 ºC
- No significant difference in viability between spores stored at room temperature vs. 4 ºC after six months



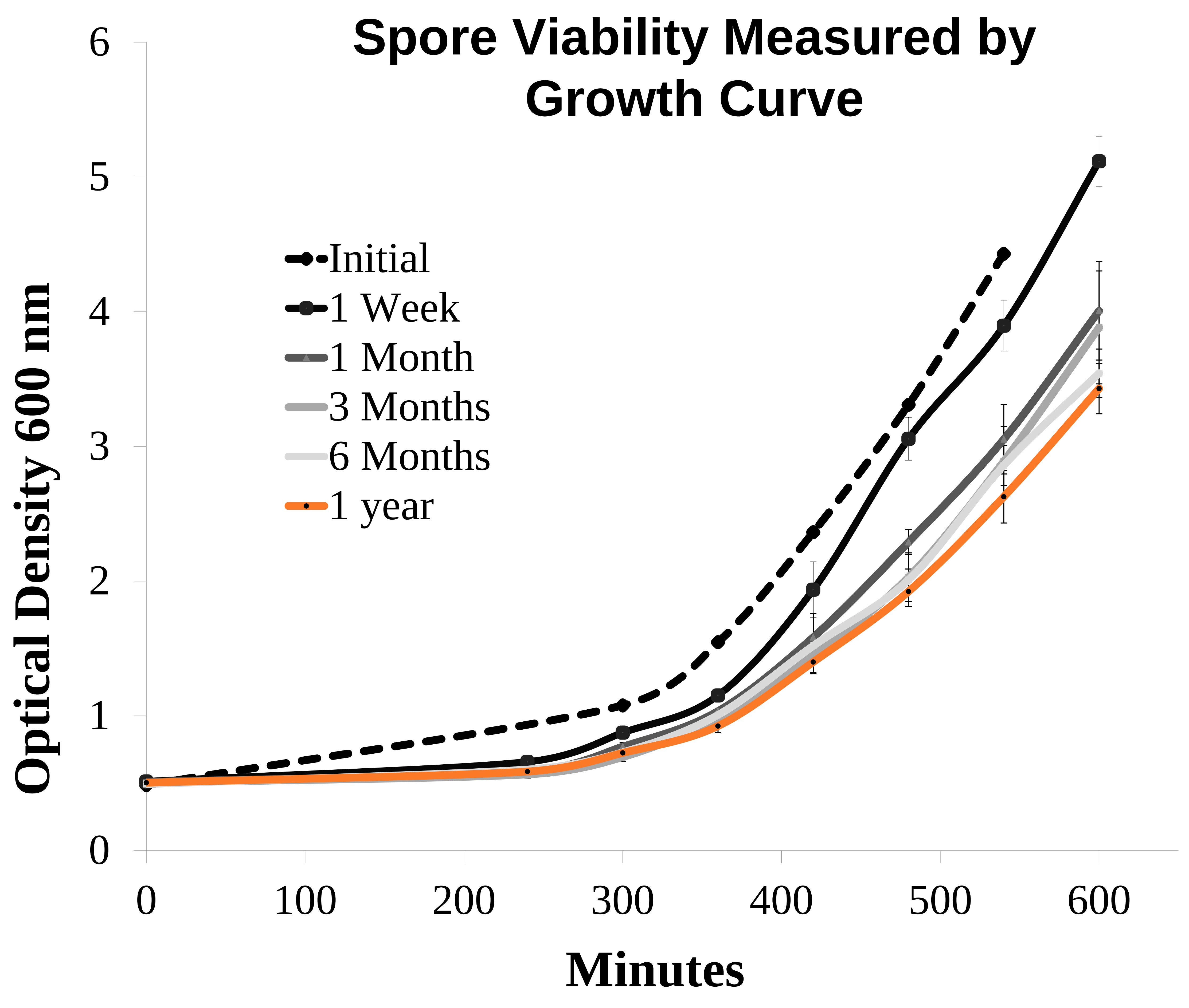
S. cerevisiae Spore Storage

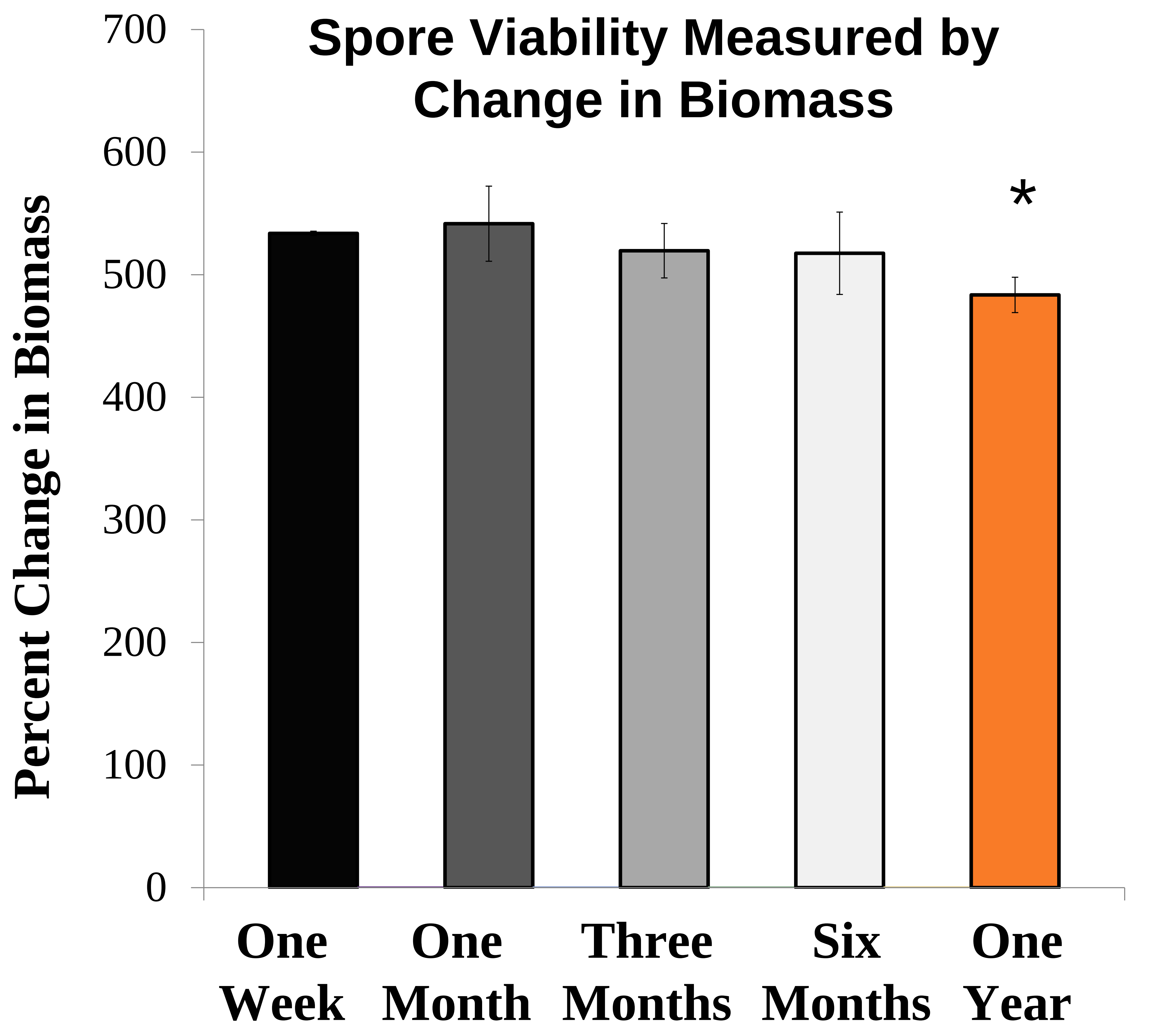
A. Sporulation at room temperature
B. Spores dehydrated in a desiccator
C. Spores dehydrated at 4 ºC
D. Spores stored in water
E. Spores dehydrated by vacuum



- No spores survived when stored in water after 6 months
- Minimal decline in viability for spores stored under all parameters

Three-year Spore Storage Study





* Represents 10% less final biomass than samples stored for one week

Conclusions from Storage Study – 1 Year

- Spores have maintained a relatively high viability over time
- After one year there has only been a 10% decline in overall final biomass
- In the event cell viability declines to undesirable levels, a higher starting biomass can be added to the package to offset cell loss over time.

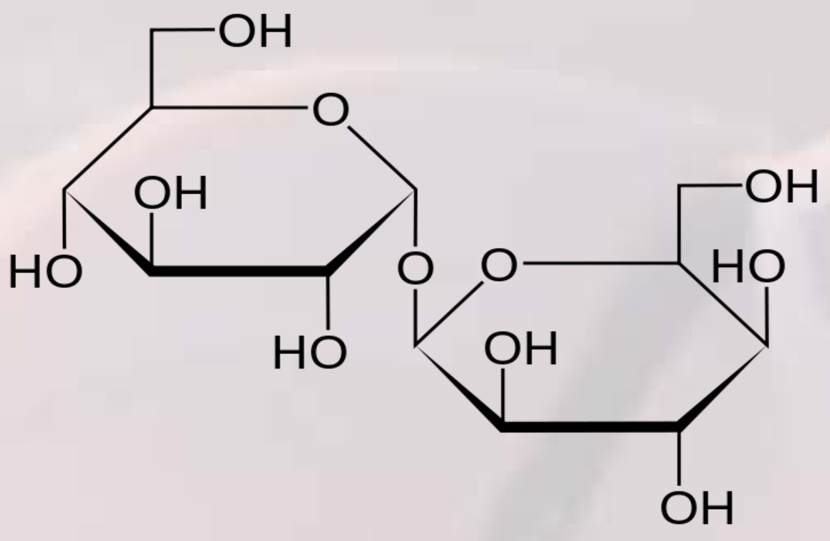
Anhydrobiotic Engineering

Trehalose

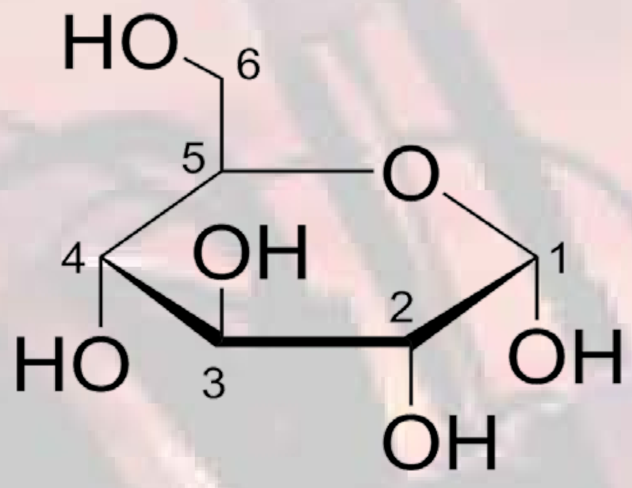
- Long-term desiccation leads to loss of molecular chaperone function
- Trehalose may act as a replacement molecular chaperone by inhibiting protein aggregation and misfolding

Traditional Pathway:

Trehalose → Glucose

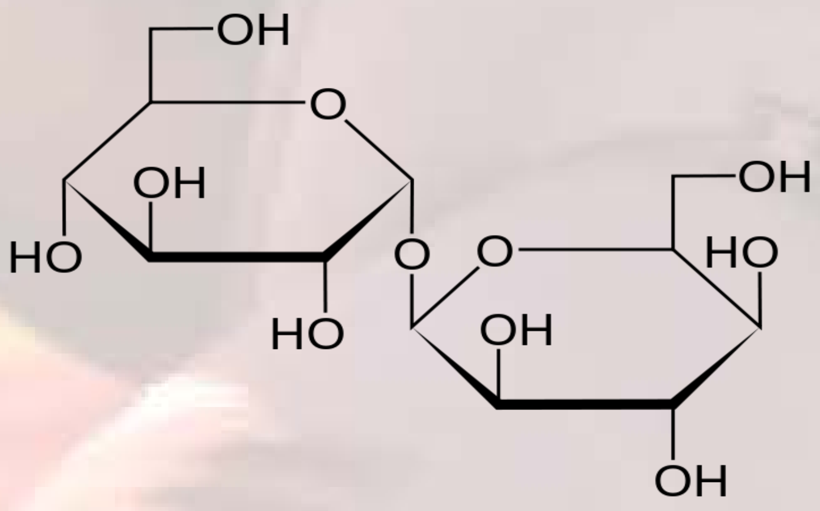


Trehalase (NTH1)

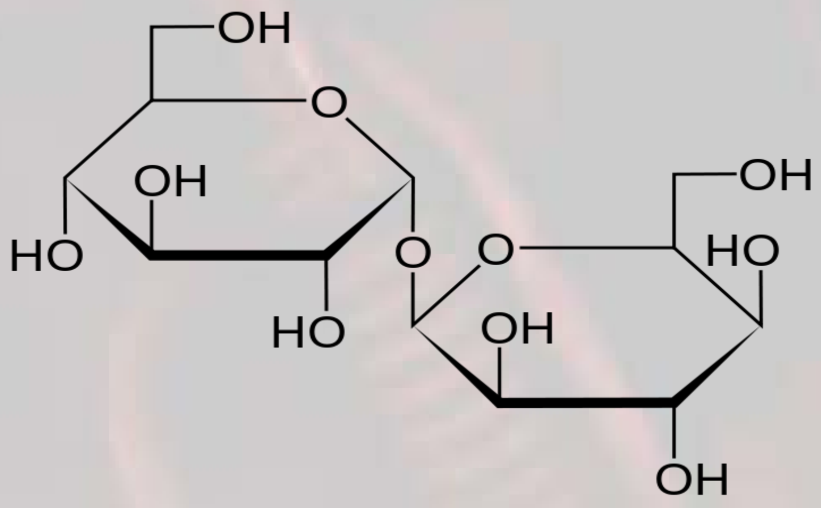


Pathway with Engineered NTH1 Knockout:

Trehalose → Increased Trehalose in Cell



Trehalase (ΔNTH1) Knockout

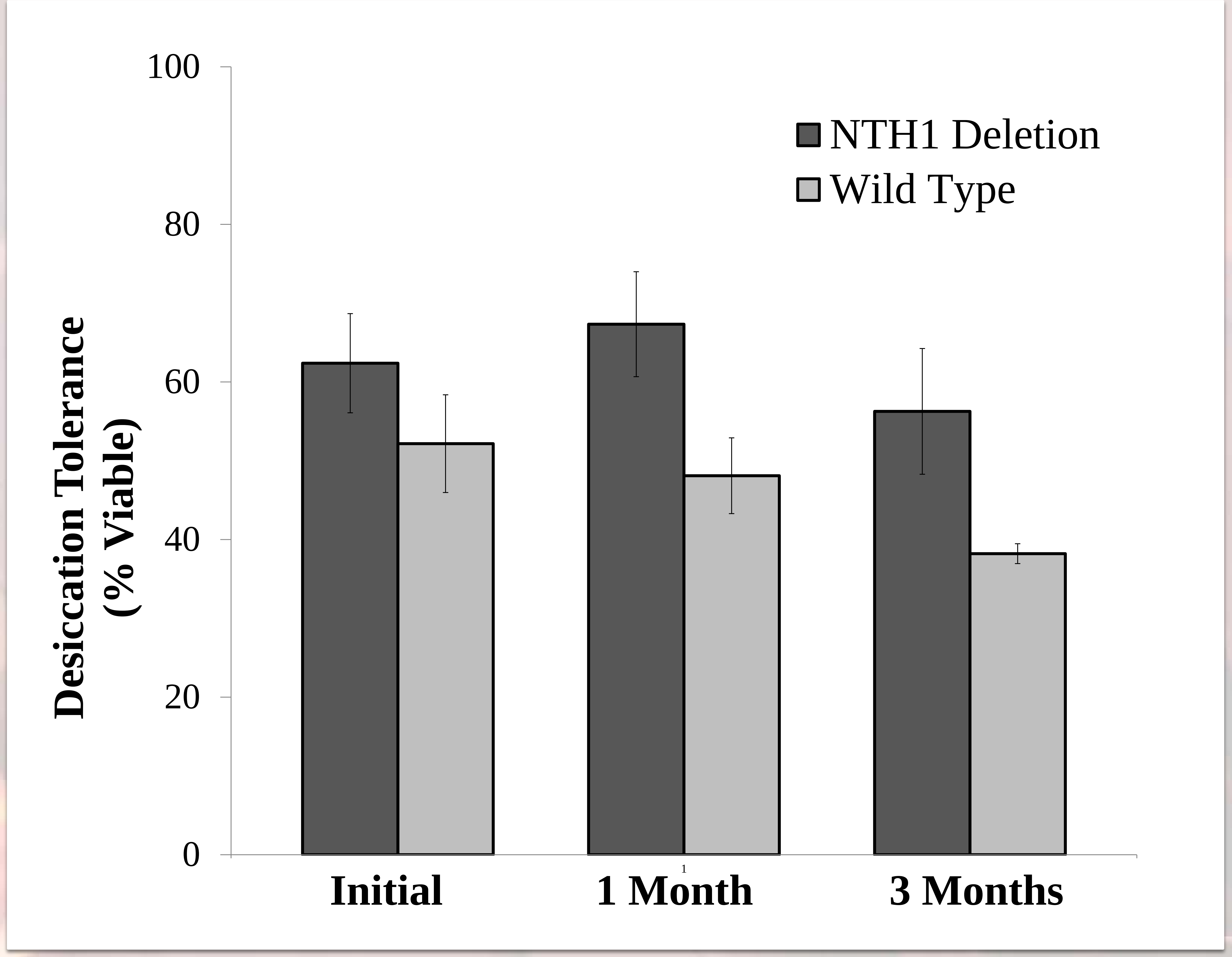


Tapia, Hugo, and Douglas E. Koshland. "Trehalose is a versatile and long-lived chaperone for desiccation tolerance." *Current Biology* 24.23 (2014): 2758-2766.

Engineering Desiccation Tolerance

- After three months the wild type *S. boulardii* strain shows a significant decline in viability compared to the NTH1 deletion strain

- Longer term data is need to verify increased desiccation tolerance over time



Summary

- *S. cerevisiae* spores have maintained high viability over one year
- Lyophilization was dropped as a drying method for spores as the freezing step is likely overly damaging
- Air-drying vegetative cells results in the highest initial viability directly after drying
- Early stationary phase appears to be the optimal time to prepare yeast for desiccation
- NTH1 knockout may increase long-duration survival of *S. boulardii* in a desiccated state although longer term storage data is needed to verify

Acknowledgements

I would like to thank the rest of my team at the NASA Ames Research Center for their contributions to this research and this paper

Funded by

NASA AES, Foundational Domain, Synthetic Biology Applications

References

[1]Cooper, M., Douglas, G. and Perchonok, M., "Developing the Nasa Food System for Long-Duration Missions," *Journal of Food Science*, Vol. 76, No. 2, 2011, pp. R40-R48.

[2]*Code of Federal Regulations*, Food and Drugs, Title 21, Vol. 2, sec. 101.9, 2016

[3]Sauer, M. et al., "Production of L-ascorbic acid by metabolically engineered *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*." *Applied and environmental microbiology,* Vol. 70, No. 10, 2004, 2004, pp. 6086-6091

[4]Yanagisawa, Y., and Sumi, H., "Natto Bacillus Contains a Large Amount of Water-Soluble Vitamin K (Menaquinone-7)." *Journal of food biochemistry,* Vol. 29, no. 3, 2005, pp, 267-277.

[5]Verwaal, R. et al., "High-level production of beta-carotene in *Saccharomyces cerevisiae* by successive transformation with carotenogenic genes from *Xanthophyllomyces dendrorhous*." *Applied and environmental microbiology,* Vol. 73, No. 13, 2007, pp. 4342-4350.

[6]Dupont, S., Rapoport, A., Gervais, P. and Beney, L., "Survival Kit of *Saccharomyces cerevisiae* for Anhydrobiosis," *Applied Microbiology and Biotechnology,* Vol. 98, No. 21, 2014, pp. 8821-8834.